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Trends in Antifungal Susceptibility among Clinical Isolates of *Candida* spp. Resistant to Antifungals and Natural Products

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Abstract

A collection of 260 *Candida* strains isolates recovered from 198 patients from 13 different medical centers in Jabalpur over a period of 5 years. These were tested for resistance to various antifungals according to the guidelines of NCCLS documents M 38-P by various methods. The isolates which were found to be resistant to synthetic antifungals were selected and subjected to antifungal testing against natural herbs and spices.Amongst all the spices and natural herbs tested garlic was found to the most effective against *Candida* strains.

Key Words: Clinical isolates, *Candida* sp., Antifungal agent

Introduction

Antimicrobial resistance surveillance serves many purposes. The most common of which is detection and tracking of resistance trends and emerging new threats[11,13,14,16]. Clinically this is important for treatment recommendations and as a means to assess the prevalent pathogens causing serious infections. The isolates collected in this program can be used to assess the activities of new antimicrobial agents and to aid in the development and validation of new susceptibility methods[11,16,10].

Over the past few decades the incidence of fungal infections has increased. The treatment of choice for infected patients remains amphotericin B, itraconazole, voriconazole etc. the appropriate susceptibility is decided by various in vitro susceptibility methods[2,12,15,20,23,24]. The studies to date that have documented the efficacies of agar based methods for the testing of susceptibilities to fluconazole or voriconazole have generally included adequate number of *Candida albicans* species but few *Candida glabrata* isolates. From susceptibility testing one can determine the resistivity of the clinical isolate to various antifungals by determining the MIC range against a sufficiently large number of isolates.

Apart from the synthetic antifungal antibiotic the naturally occurring herbal products and spices are known to have powerful antifungal properties. Research has proved their antifungal activities. Herbs like goldenseal, myrrh, walnut, licorice, lemongrass and spices like turmeric, cinnamon, clove, ajwain and medicinal plants like neem, tulsi, eucalyptus etc. have a great antifungal profile. Even the combination of these works in synergy and gives better results then a single herb[1,19,22].

Materials and Methods

Organism: A total of 260 clinical isolates of *Candida species* obtained from 13 medical centers of Jabalpur were tested .The collection included 175 isolates of *C. albicans*; 43 isolates of *C. tropicalis*; 12 isolates of *C. krusei*; 17 isolates of *C. parapsilosis* ; 5 isolates of *C. glabrata* ; 8 isolates of *C. guilliermondii*. Isolates were identified using conventional methods, growth in CHROM Agar media and using Atlas of fungi. They were stored as water suspension until used. Prior to testing, each isolate was passaged at least twice on SDA with chloramphenicol to ensure purity and viability.

Antifungal agents: Voriconazole (Pfizer), fluconazole (Pfizer), itraconazole (Jansen), flucytosine (Sigma), amphotericin B (Hi media). Serial two fold dilutions were prepared exactly as outlined in NCCLS document M 27A (17). Final dilutions were prepared in RPMI 1640 medium (Sigma) buffered to pH 7.0 with 0.165M morpholine propane sulphonic acid (MOPS) buffer (Sigma). The dilutions were kept in Eppendorf's tubes until used and stored at -4°C in Quick freezer.

Natural products: Turmeric, cinnamon, clove, ginger, garlic, neem, tulsi were used for the present study. The natural products were dried, powdered and then alcoholic extracts were prepared. The discs of natural products were prepared by soaking the discs in alcoholic extracts for a period of 24 hours and then dried in air. Two fold dilutions were prepared from powdered form in RPMI 1640 medium for broth dilution method.

Susceptibility testing: Reference antifungal Susceptibility testing of *Candida species* was performed by disc diffusion method as described by Barry et al (5) and BMD method as described in NCCLS documents (17). MIC's were determined with RPMI 1640 agar. An inoculum suspension adjusted to the turbidity of a 0.5 McFarland standard (~10^6cells/ml), and incubation at 35°C for 48 hours. MIC interpretive criteria were those published by Rex et al and the NCCLS document M 27 A and were as follows: susceptible, MIC $\leq$8µg/ml; susceptible-

dose dependent, MIC = 16 to 32µg/ml; resistant, MIC ≥ 64µg/ml. The interpretive criteria for disc test were those published by Barry *et al*.[5] and the NCCLS document M 27 A (17): susceptible, zone diameter of ≥ 19mm; susceptible-dose dependent, zone diameter of 15 – 18 mm; resistant, zone diameter of ≤ 14mm.

Quality control: QC was performed for BMD & disc diffusion method in accordance with NCCLS document M 27 A[17] by using *C. albicans* 3809 as reference culture.

Analysis of results: The diameters of the zone of inhibition for the test antifungals were measured in mm by disc diffusion method and their respective BMD MIC's were also studied. The errors were identified as susceptibility by one method and resistivity by other method.

Results and Discussion

The species distribution of *Candida* isolates in present investigation is summarized in Table 1. These isolates were obtained during the course of *Candida* surveillance studies and represent a total of 260 clinical isolates. Most of the species of *Candida* isolated in present study have been previously reported to cause serious infections in humans[19,20,21, 22].

Table 1: Species distribution of *Candida* isolates

Species isolated	No. of isolates	% of isolates
Candida albicans	175	67.32
Candida tropicalis	43	16.54
Candida krusei	12	4.61
Candida parapsilosis	17	6.54
Candida glabrata	5	1.92
Candida guilliermondii	8	3.07
Total	260	100

Several species such as *C. krusei*, *C. guilliermondii*, and *C. parapsilosis* have been reported to express resistance to antifungal agents. Thus, it is evident that these non albicans species of *Candida* may be considered as opportunistic pathogens and that these may be responsible in posing resistance problems for the currently used antifungal agents. From the table it is clear that the most frequently encountered *Candida* species is *C. albicans* followed by *C. tropicalis, C. parapsilosis, C. krusei* etc. The antifungal susceptibilities of the isolated *Candida species* are summarized in Table 2.

Table 2: In vitro susceptibility of *Candida* species against various antifungals

Antifungals	AMP B			5 FC			FLU			VORI			ITRA		
Susceptibility	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Disc diffusion	203	41	16	240	09	11	135	65	60	198	32	30	146	88	26
Broth dilution	196	53	11	168	75	17	112	98	50	206	28	26	185	69	06

AMP B –Amphotericin B, 5FC- 5 Fluocytosine, FLU-Fluconazole, VORI-Voriconazole, ITRA-Itraconazole.
S-Sensitive; I-Intermediate; R-Resistant.

Table 3: In vitro susceptibility of *Candida* species against alcoholic extract of various natural herbs and spices measured as zone of inhibition in cms

S. No	Clinical fungal isolate	No. of isolate	neem	tulsi	ginger	Garlic	onion	Aloe vera
1	*Candida albicans*	16	1.4	0.4	1.0	1.6	1.8	0.6
2	*Candida tropicalis*	12	1.2	-	-	1.8	1.3	0.5
3	*Candida krusei*	05	1.4	0.4	1.2	1.5	1.2	-
4	*Candida parapsilosis*	04	1.2	-	1.0	1.6	1.3	0.5
5	*Candida glabrata*	07	1.2	-	1.0	1.6	1.4	0.4
6	*Candida guilliermondii*	03	1.0	-	1.2	1.4	-	0.6

The clinical isolates resistant to antifungal were selected and subjected to the in vitro sensitivity against the natural products .The sensitivity of the strains was measured as zone of inhibition in cms. The sensitivity of the clinical isolates to natural extracts is expressed in table 3.The mean value of the number of isolates for each strain was studied. From the table it is clear that the resistant strains of clinically isolated *Candida spp.* are sensitive to spices and herbs used as a supplement in diet. The most effective amongst all the spices and herbs is garlic (*Allium sativum*), followed by onion(*Allium cepa*), neem (*Azadirachta indica*), ginger (*Zingiber officinale*) and *Aloe vera*. Amongst all the *Candida* strains tested the neem extract was found to be effective against *C. albicans* and *C. krusei* followed by *C. tropicalis*, *C. parapsilosis* and *C. glabrata*. Tulsi (*Ocimum sanctum*) extract was found to be least effective. The antifungal activities of some spices and herbs have been reported by Anupam *et al*. 2005. The results of the present investigation are according to those obtained by Anupam et al. It is clearly illustrated from the table that the common spices used in our daily life and the extracts of natural herbs effects

the fungal growth adversely and therefore might be considered to control fungal diseases. The bioactive compounds in these plant extracts must be analyzed, purified and then can be used as drug for controlling fungal pathogens without the development of drug resistance in pathogens and also without any side effects.

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